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Cells under endoplasmic reticulum stress show increased expression of GADD153, a pro-apoptotic C/EBP family transcription factor. Cellular stress also induces the activity of the transcription factor NF-kB, which promotes cell survival. We showed inhibition of GADD153 mRNA expression by the p65 transactivating subunit of NF-kB and proposed that of cells expressing constitutively active NF-kB may be predisposed to transformation following cellular stress due to p65-mediated inhibition of GADD153 expression. We generated immortalized mammary epithelial cells MCF-10A overexpressing p65. p65 overexpressing cells showed hallmarks of epithelial to mesenchymal transition (EMT) including reduced expression of E-cadherin and Occludin and increased expression of Vimentin and stromelysin-1. The expression pattern of c/EBPβ was altered in p65 overexpressing cells compared to parental cells. Parental MCF10A cells cultured in matrigel organized into polarized lobular acinar structures, whereas MCF10A/p65 cells formed disorganized structures, similar to ErbB2-transfected MCF10A cells. Interestingly, the expression levels of the transcription repressors ZEB-1 and ZEB-2 but not Snail, all of which are involved induction of EMT, were elevated in MCF10A/p65 cells. P65 reduced the expression of deltaNp63, a marker for myoepithelial cells. We have also identified the transcription repressor oncoprotein Gfi-1 as the transcriptional target of NF-κB and Gfi-1 reduced GADD153 expression. These results suggest that NF-κB promotes breast cancer initiation through EMT, inducing the expression of oncoprotein such as Gfi-1, and

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INTRODUCTION:

Understanding the mechanism of breast cancer initiation is critical for developing chemoprevention strategies. Normal mammary epithelial cells from which cancer usually originate have limited life span. Immortalization is the first step that leads to continuous growth of mammary epithelial cells (1). Cell cycle regulatory, anti-apoptotic and pro-apoptotic proteins play a significant role in immortalization process (1). Immortalized cells attain cancerous growth properties (transformation) due to additional mutations that lead to either loss of tumor suppressor genes or activation of oncogenes. The transcription factor NF-κB promotes both immortalization and transformation by controlling the expression of cell cycle regulatory, anti-apoptotic and pro-apoptotic genes (2). NF-κB is usually sequestered in the cytoplasm of resting cells through its association with inhibitor of kappaB proteins and translocates to nucleus upon exposure of cells to cytokines and growth factors (3). NF-κB then binds to response elements and induces the expression of genes involved in invasion, metastasis and chemotherapy resistance. We and others have shown that NF-κB is constitutively active in breast cancer and is responsible for overexpression of several anti-apoptotic genes as well as repression of the pro-apoptotic gene GADD153/CHOP (4-7). GADD153 is induced when DNA is damaged or cells are under stress. Depending on the extent of damage, cells either repair DNA damage and survive or die. GADD153 is believed to promote death of cells with severe damage, thereby limiting accumulation of cells with mutations (8). Thus, GADD153 is likely to play a role in preventing breast cancer initiation. Because NF-κB reduces GADD153 expression, it is possible that cells that contain constitutively active NF-κB will survive after DNA damage. Cells with damaged DNA are more prone for transformation. This award is to test this possibility. There are three aims in this proposal: 1) To determine whether inhibition of GADD153 by NF-κB is essential for survival and/or transformation of MCF-10A cells when exposed to MMS or grown under nutrient-deprived condition. 2) To determine whether inhibition of GADD153 by NF-κB leads to altered activity of the transcription factor C/EBPβ and differentiation of MCF-10A cells. 3) To determine the influence of p53 on the anti-apoptotic function of NF-κB in MCF-10A cells grown under nutrient-deprived condition or exposed to MMS.

BODY:

Specific Aim I:

Task 1:(months 1-5) Establish MCF-10A cells overexpressing p65NLS50 or ras and characterize them with respect to constitutive NF-kB activity.

Results: This task is completed as reported in last years report

Task 2: (Months 6-8) Determine MMS-inducible and nutrient-deprivation inducible expression of GADD153 in MCF10/p65NLS50 cells by Northern and Western blots. Determine apoptosis by Annexin V labeling, PARP cleavage and DNA laddering.

Results: Initial cell viability experiments were carried out using the MTT assay and clonogenic assays. MCF-10A/p65NLS50 cells treated with MMS (0 to 10 mM) for 3 to 24 hrs did not show a significant difference in cell viability, as measured by MTT assay

compared to vector control cells. Unlike in cancer cells, serum starvation did not induce GADD153 in MCF-10A cells.

Task 3: Months (9-15) determine the susceptibility of MCF-10A, ras and p65NLS50 cells to MMS-induced transformation. This will be achieved by growing cells in soft agar and matrigel.
Results: MCF-10A cells plated on a Matrigel form a highly organized acinar structure and have been used extensively as a model system to study the formation and maintenance of glandular architecture *in vitro*. These acinar structures consist of a hollow lumen surrounded by a single layer of cells, with the hollow lumen formed through a selective apoptosis of centrally located cells (9). Oncogenes such as ErbB2 prevent apoptosis and induce proliferation of the centrally located cells, which leads to the formation of a highly disorganized structure (10). As expected, the parental MCF-10A/pQ cells cultured in matrigel formed polarized acinar structures (Fig. 1). In contrast, MCF-10A/p65 cells formed disorganized structures that are reminiscent of those formed by MCF-10A cells overexpressing the ErbB2 oncogene (10). Because ErbB2 has been shown to activate NF-κB (11), these results suggest that disruption of the acinar structure by ErbB2 may be mediated through NF-κB. Thus, NF-κB activation alone is sufficient for MCF-10A cells to show a transformed cell phenotype in matrigel.

The following results with MCF10/p65NLS50 cells reveal a unique function for NF-κB in breast cancer progression.
p65 expression induces an altered phenotype in MCF-10A cells: In the last report we indicated that NF-κB induces EMT phenotype. We have performed a series of RT-PCR analysis of epithelial and mesenchymal markers in MCF-10A/pQ and MCF-10A/p65 cells. MCF-10A/p65 cells showed reduced expression levels of epithelial markers, including E-cadherin, occludin, desmoplakin, MUC 1/episialin, and keratin 18 (K18), compared to MCF-10A/pQ control cells (Fig. 2A). In contrast, expression of the mesenchymal markers, vimentin, and stromelysin-1/MMP3 (matrix metalloproteinase-3) were increased. In addition, fibronectin expression was increased. In contrast, the myoepithelial marker α-smooth muscle actin (αSMA) (12) was expressed at similar levels in both cell lines (Fig. 2B).

Increased cellular motility of MCF-10A/p65 cells. Increased motility is another hallmark of EMT (13).We used wound assay to compare motility of MCF-10A/pQ and MCF-10A/p65 cells. Over a period of 24 hrs, MCF-10A/p65 cells showed a faster rate of motility compared to MCF-10A/pQ control cells (Fig. 3).

Induction of ZEB-1 and ZEB-2 expression by NF-κB. To identify genes downstream of NF-κB that may play a role in EMT, we performed RT-PCR and Northern blot analysis of transcriptional regulators involved in EMT (14, 15). MCF-10A/p65 cells showed increased expression of the transcriptional repressors ZEB-1 and ZEB-2 but not other transcriptional repressors Snail-1, -2 or -3. In fact, Snail-1 expression was slightly decreased in MCF-10A/p65 cells (Fig. 4A). These results are distinct from the results in drosophilae, where the NF-κB homologue dorsal induces EMT through Snail (16). There was no difference in the expression levels of CtBP-1 and CtBP-2, corepressors required

for ZEB-1 and ZEB-2 mediated transcriptional repression (data not shown, (17)). Induction of ZEB-1 and ZEB-2 by NF-κB is not restricted to MCF-10A cells because activation of NF-κB through treatment with IL1-α, TNF-α or TPA in the breast cancer cell line MBA-MD231 led to increased ZEB-1 and ZEB-2 expression (Fig. 4B). Our repeated attempts to show that ZEB-1 and ZEB-2 are responsible for NF-κB-mediated EMT using siRNA technique were not successful. Similarly, we were not successful in generating MCF-10A cells overexpressing ZEB-2. Nonetheless, based on the previously reported role of these proteins in maintaining EMT phenotype (15), we suggest that ZEB-1 and/or ZEB-2 are responsible for NF-κB-mediated EMT.

Specific aim II:

Task 1: Determine the DNA binding pattern of C/EBPβ and C/EBPβ:GADD153 heterodimers in various cell types by EMSA.

Results: Three isoforms of C/EBPβ are expressed in mammary gland. Normal mammary gland expresses full-length C/EBPβ-1 whereas breast cancers express shorter C/EBPβ-2 (18). C/EBPβ1 and C/EBPβ2 are derived from a same transcript utilizing different in-frame initiation codon and differ only by 24 amino acids. Overexpression of C/EBPβ-2 alone is sufficient for EMT of MCF-10A cells (19). We observed a switch in C/EBPβ isoforms upon overexpression of p65. While the MCF-10A/pQ cells expressed ~50 kDa protein, which corresponds to C/EBPβ-1 isoform, MCF-10A/p65 cells expressed C/EBPβ protein of ~46 kDa (Fig. 5). This is most likely C/EBPβ-2 isoform. Overexpression of C/EBPβ-2 isoform has been shown to increase cyclin D and induce EMT in MCF-10A cells (19). Increased cyclin D1 expression and EMT is observed in MCF-10A/p65 cells. Thus, it is possible that, apart from ZEB1 and ZEB-2, altered expression of C/EBPβ is responsible for EMT of MCF-10A/p65 cells.

Task 2: Determine the expression levels of C/EBPβ and GADD153-responsive genes in various cell types by transient transfection, Northern and Western blots.

Results: We have measured expression level of β-casein in MCF-10A/pQ and MCF-10A/p65 cells. Consistent with changes in C/EBPβ expression pattern, we observed increased expression of β-casein in MCF-10A/p65 cells compared to MCF-10A/pQ cells (Fig. 5)

Task 3: Months: determine the differentiation pathway in various cell types in response to lactogenic stimulation. This will be achieved by measuring β-casein and WAP expression in response to prolactin treatment.

Results: Without any prolactin treatment, we have observed significant change in differentiation program in MCF-10A/p65 cells as outlined below.

Because MCF-10A/p65 cells showed marked difference in their morphology, we examined whether these changes are associated with changes in the expression pattern of genes that are considered as progenitor cell markers. Cytokeratin 5 (CK5) is considered as a marker for progenitor cells that can differentiate to either glandular cells (CK8/18+) or myoepithelial cells (SMA+) cells (12). P63, a p53 homologue, is considered as a marker for myoepithelial cells (20). P63 is expressed as several different isoforms; TAp63 and ΔNp63 are the major isoforms derived from alternative promoters. While

TAp63 has a transactivation domain and can activate gene expression through p53 response element, ΔNp63 can dominantly inhibit p53 function. ΔNp63 is most commonly expressed isoform in myoepithelial cells (21). Recent knockout studies have shown that while TAp63 promotes commitment of the ectoderm to epithelial lineages, induces proliferation, and suppresses the differentiation of the committed progenitor cells ΔNp63 isoform promotes terminal differentiation (22, 23). Microarray analysis indicated that TAp63 and ΔNp63 regulate the expression of different set of genes (24). In addition, 12 genes show an inverse correlation between isoforms, suggesting that these isoforms can have opposing functions.

CK5 expression was observed in MCF-10A/pQ cells but not MCF-10A/p65 cells (Fig. 6A), which suggests that NF-κB alters the progenitor cell properties of mammary epithelial cells. Examination of p63 gave rise to starling results. ΔNp63 isoform expression was restricted to MCF-10A/pQ cells (Fig. 6B). MCF-10A cells exposed to TNF, IL-1 and TPA showed reduced levels of ΔNp63, which confirms NF-κB-dependent repression of ΔNp63 (Fig. 6C). Preliminary results indicates that repression of ΔNp63 by NF-κB is mediated by ZEB-2. These results clearly suggest that NF-κB can alter progenitor cell properties of mammary epithelial cells.

Specific aim III

Task 1: Establish cells overexpressing p53 dominant-negative mutants and characterize them for p53 activity. Stable cell lines overexpressing p53V144A will be established.

Results: We have generated MCF-10A cells overexpressing dominant negative p53 (Fig. 7). Unfortunately, this particular variant of p53 failed to function in a dominant negative manner in MCF-10A cells because doxorubicin-inducible activation of p53-responsive genes (p21 and Bax) were same in parental and dominant negative p53 overexpressing cells (Fig. 7). Therefore, we will use commercially available retrovirus vectors with siRNA against p53 to generate MCF-10A cells lacking p53.

Task 2: Determine MMS sensitivity, NF-κB activity and GADD153 expression in various cell types.

Task 3: Determine the MMS-induced transformation frequency of cells that express p53 dominant negative mutant.

Results: We will initiate these studies once we establish MCF-10A cells overexpressing p53 siRNA.

NF-κB induces the oncoprotein Gfi-1, which represses GADD153.

In the last report, we showed that ~40 base pair region in the 5' untranslated region of GADD153 contains the element required for repression by NF-κB. This element contained a binding site for the transcriptional repressor Gfi-1. Gfi-1 is an oncoprotein involved in lymphoma (25). To determine whether Gfi-1 is induced by NF-κB, we examined Gfi-1 expression in parental MD-231 cells with retrovirus vector alone and MD-231 cells overexpressing IκBα super-repressor, which reduces NF-κB activity by RT-PCR followed by Southern analysis with an nested primer probe. Gfi-1 expression was lower in cells overexpressing IκBα super-repressor (Fig. 8A). In transient transfection assays, Gfi-1 reduced GADD153 promoter activity (Fig. 8B). These results

indicate that NF-κB represses GADD153 through induction of Gfi-1. NF-κB-mediated repression of GADD153 is cell type specific because Gfi-1 is expressed in a tissue restricted manner (25).

KEY RESEARCH ACCOMPLISHMENTS:

- MCF-10A cells overexpressing p65 subunit of NF-κB show an EMT phenotype.
- EMT correlates with NF-κB-dependent expression of EMT-associated transcription repressors ZEB-1 and ZEB-2.
- MCF-10A cells overexpressing p65 show altered expression pattern of progenitor cells markers including CK5 and ΔNp63.
- NF-κB alters the expression pattern of C/EBPβ isoforms.
- The oncoprotein Gfi-1 is induced by NF-κB, which may play a role in NF-κB-mediated repression of GADD153.

REPORTABLE OUTCOMES:

Abstract: Hui Lin Chua, Sunil Badve and **Harikrishna Nakshatri**. NF-kB induces epithelial to mesenchymal transition phenotype to immortalized mammary epithelial cells. NF-kB: from bench to bedside. Keystone symposia, Snow Bird, Utah (January 10-15, 2004).

CONCLUSIONS: Most of the work described in the original proposal has been completed. We have received one-year no-cost extension to complete remaining studies. Although the influence of NF-κB in regulating GADD153 expression in MCF-10A cells appears to be limited because these cells do not express Gfi-1, the transcription repressor required for NF-κB-mediated repression of GADD153, NF-κB has changed the differentiation program of MCF-10A cells. NF-κB-inducible genes ZEB-1, ZEB-2, p63 and Gfi-1 may be responsible for altered differentiation program. In particular, we observed NF-κB induced EMT of these cells. Recent studies suggest that EMT creates non-malignant stroma, which can alter the behavior of adjoining normal epithelial cells as well as cancer cells (26). It is possible myoepithelial cells with constitutive NF-κB undergo EMT and support growth, invasion and metastasis of adjoining cancer cells. Our preliminary studies have revealed that MCF-10A/p65 cells are resistant to TGFβ. TGFβ functions as tumor suppressor at the initial state of cancer but promotes invasion and metastasis at later stage (27). Therefore, NF-κB activation may be involved in switching TGFβ from tumor suppressor to tumor promoter.

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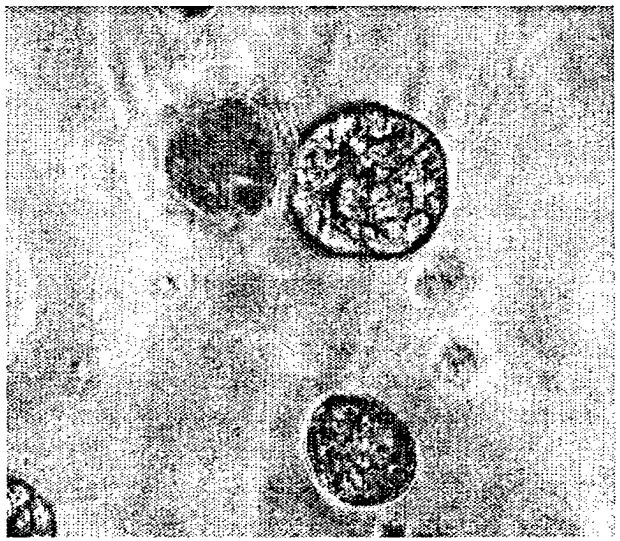
MCF10A/pQ(A)



MCF10A/p65(A)

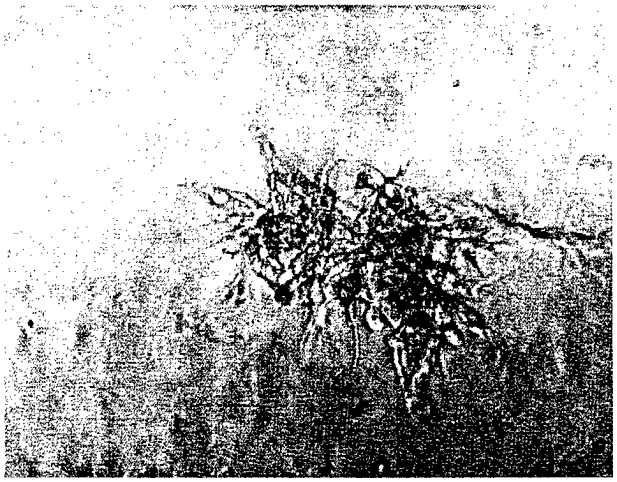


Fig. 1. p65 expression induces an altered three-dimensional structure in MCF10A cells. Cells were cultured in a three-dimensional matrix consisting of Matrigel for up to 20 days. MCF10A/pQ cells were observed to form lobular acinar structures, whereas MCF10A/p65 cells formed disorganized structures. Cells cultured to Day 15 are shown.

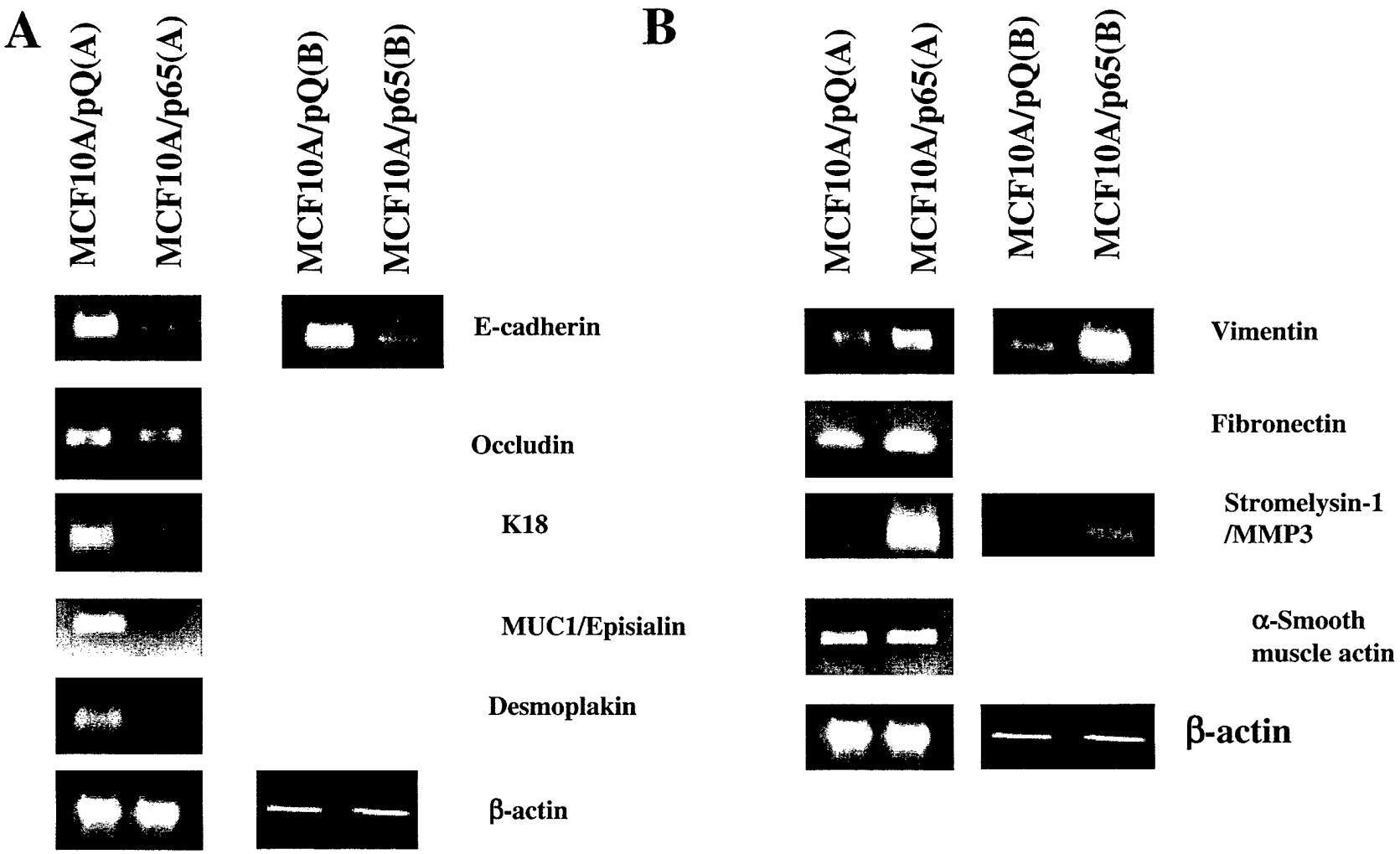


Fig. 2. p65 expression alters the expression of epithelial markers in MCF10A cells. RT-PCR analysis of MCF10A/p65 cells showed (A) reduced levels of epithelial markers including E-cadherin, Occludin, MUC1/episialin and desmoplakin compared to MCF10A/pQ control cells. (B) In contrast, MCF10A/p65 cells showed higher expression levels of mesenchymal markers including vimentin, fibronectin and stromelysin-1, compared to MCF10A/pQ control cells. In addition, smooth muscle actin was expressed at similar levels in both cell lines.

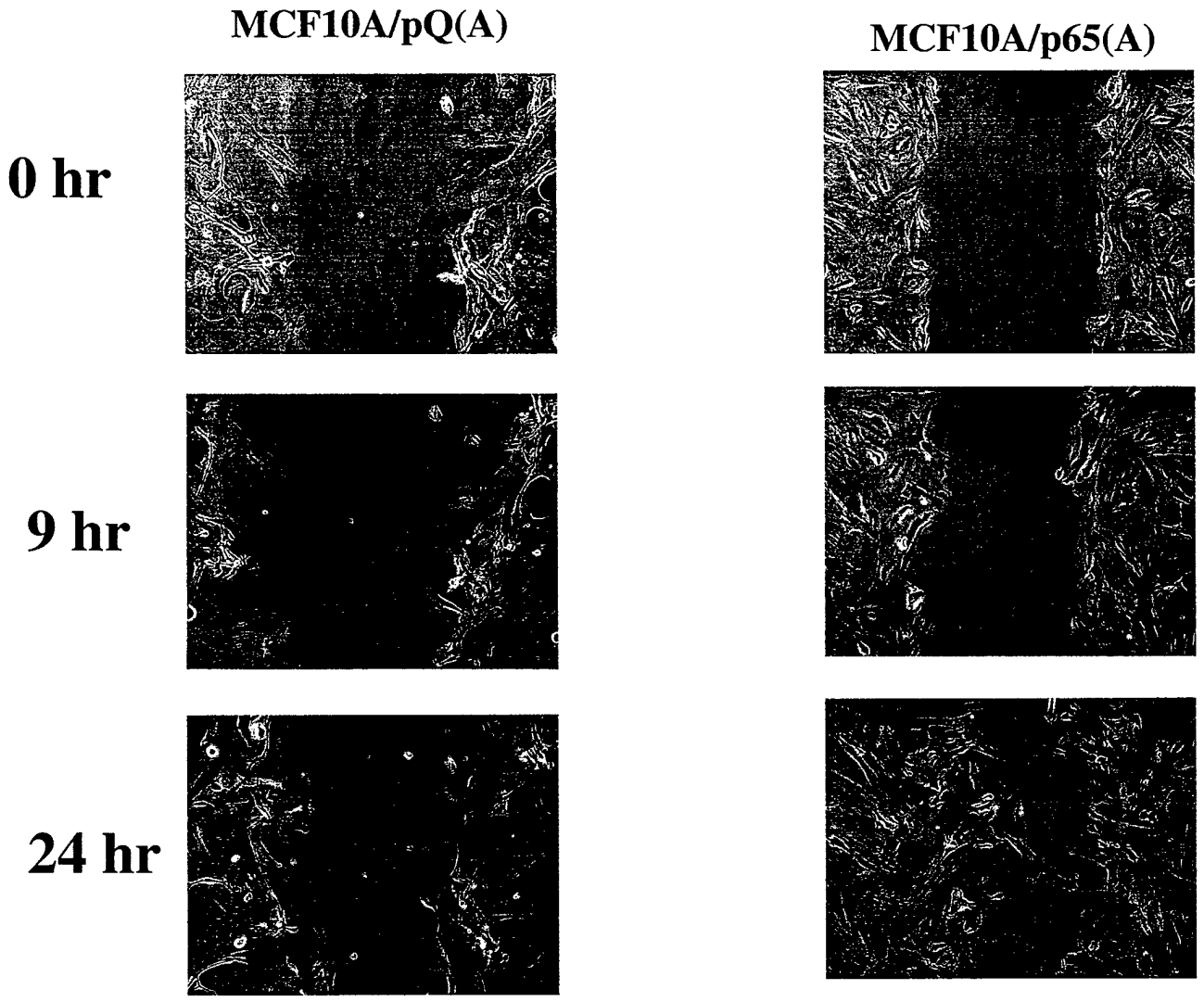


Fig. 3: MCF-10A/p65 cells are more motile than MCF-10A/pQ cells

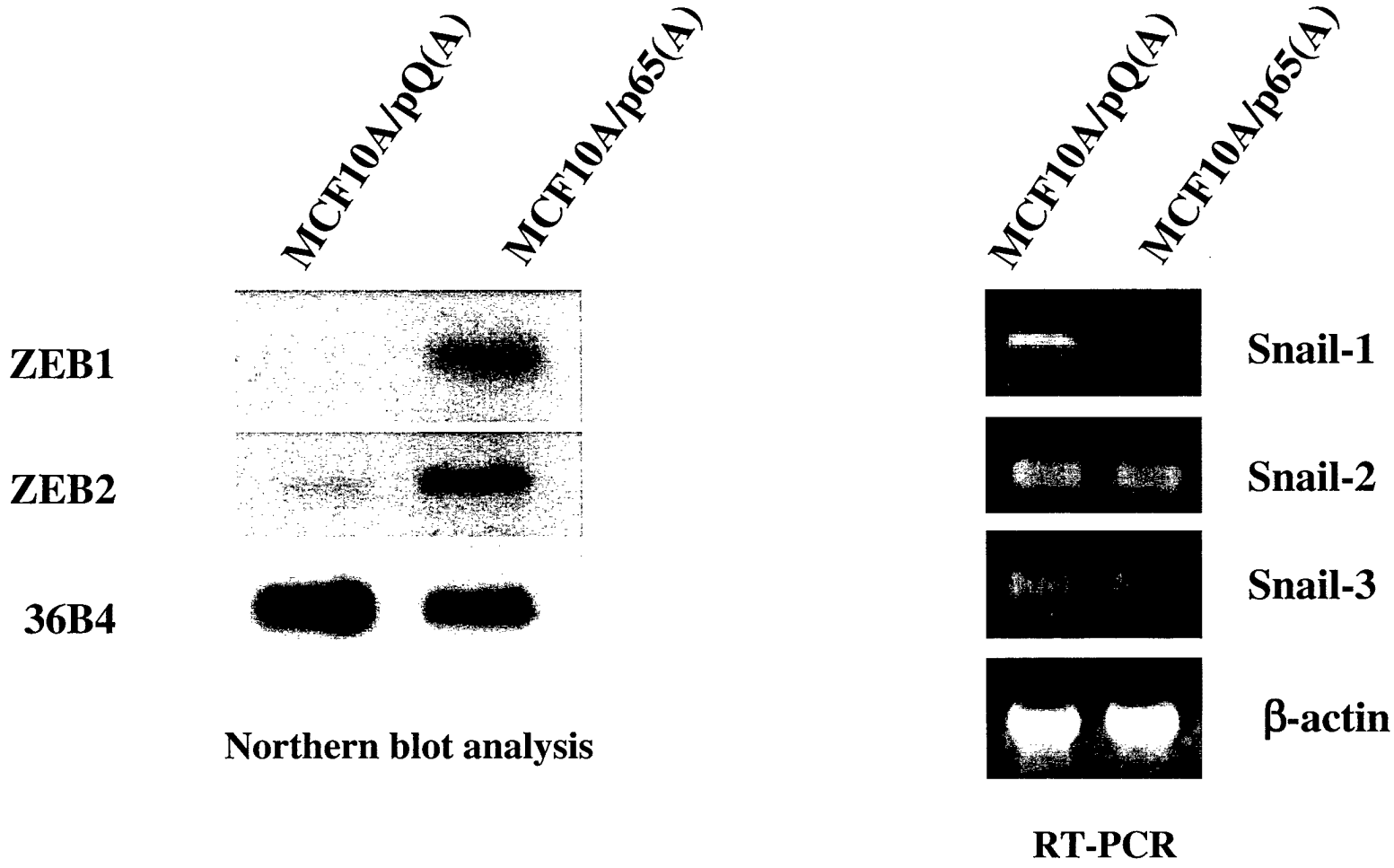


Fig. 4(A). Induction of ZEB1 and ZEB2 expression by NF-κB. (A) MCF10A/p65 cells showed increased ZEB1 and ZEB2 expression levels compared to MCF10A/pQ control cells by Northern blot analysis. In contrast, other transcriptional repressors, namely the Snail family members were expressed at similar levels in both cell lines, determined by RT-PCR analysis.

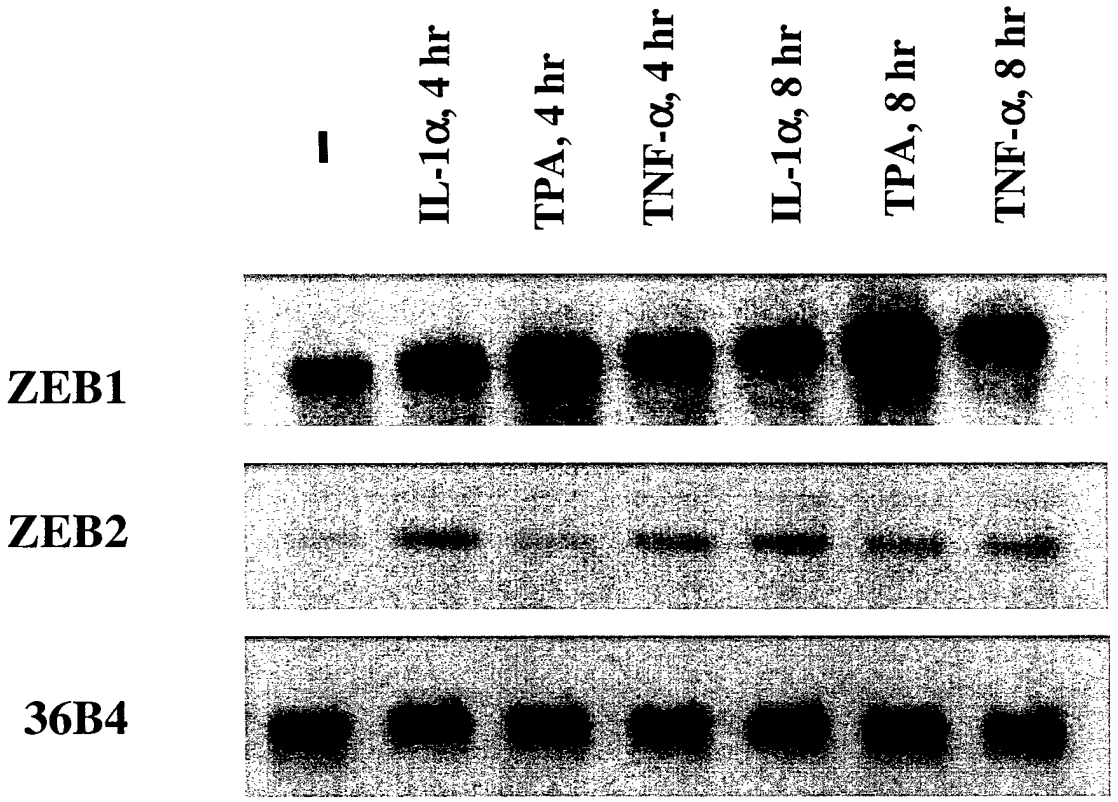
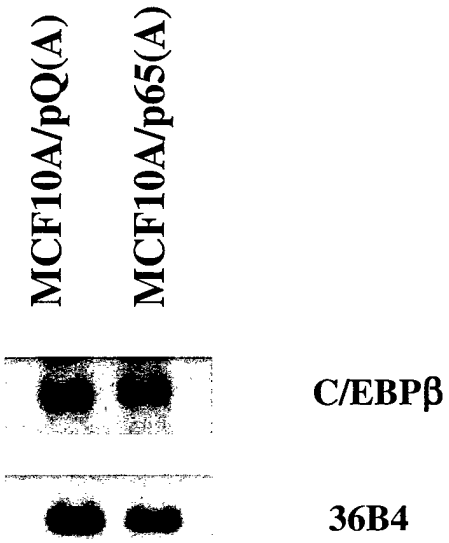
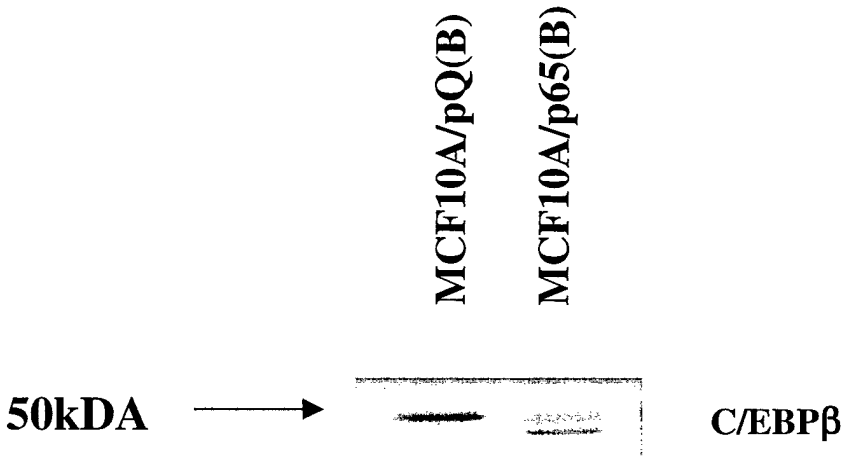


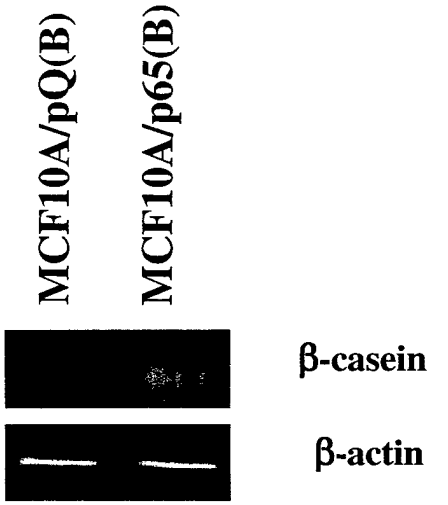
Fig. 4(B). Treatment of the MDA-MB-231 breast cancer cell line with inducers of NF-κB, IL1-α, TNF-α or TPA led to increased expression of ZEB1 and ZEB2, determined by Northern blot analysis.



Northern blot analysis



Western blot analysis



RT-PCR analysis

Fig.5: NF-κB alters C/EBPβ protein expression pattern and increases β-casein expression

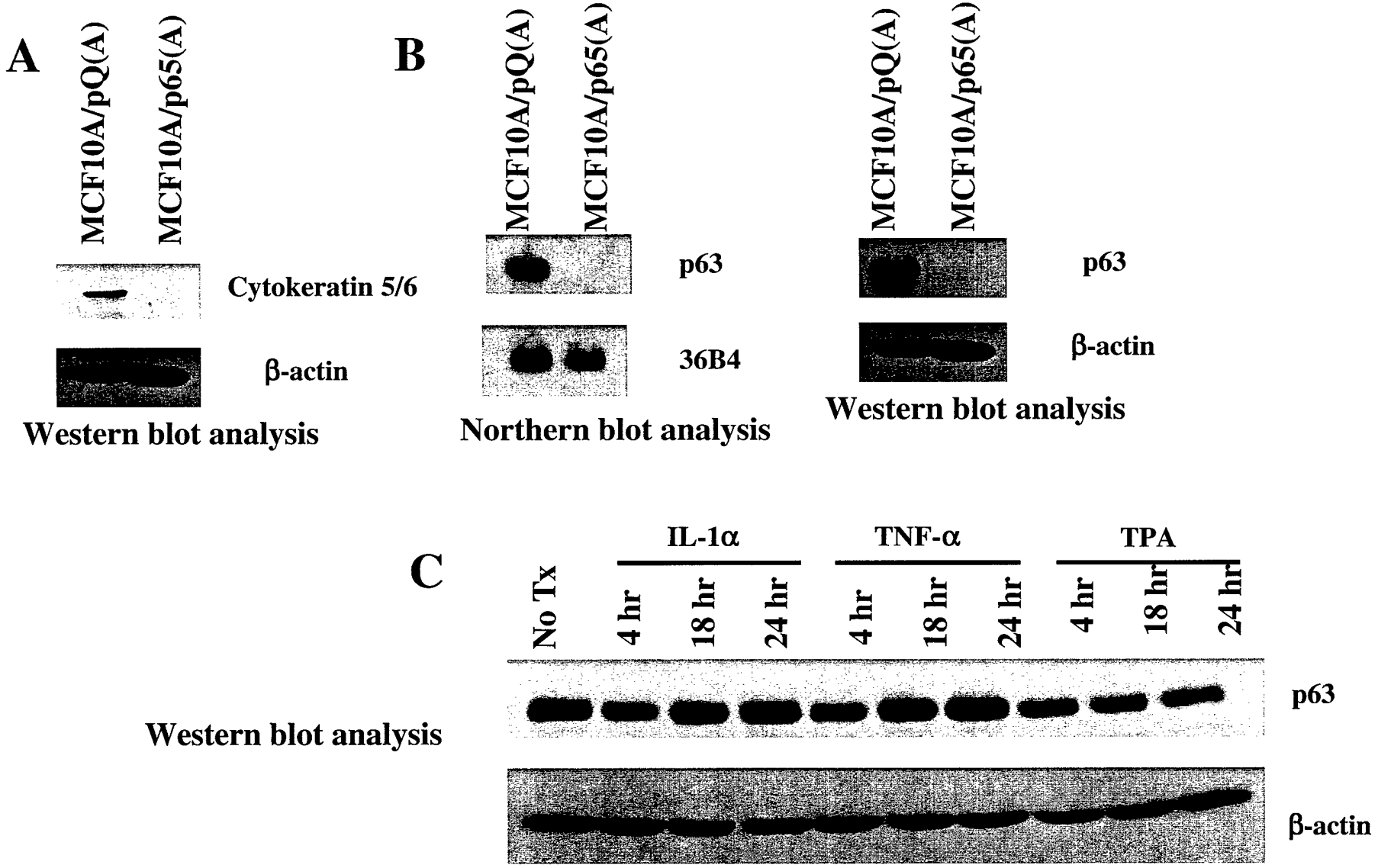


Fig. 6: NF-κB inhibits CK5/6 (A) and p63 (B and C) expression. RT-PCR analysis confirmed that MCF-10A cells express ΔNp63 but not TAp63 (data not shown)

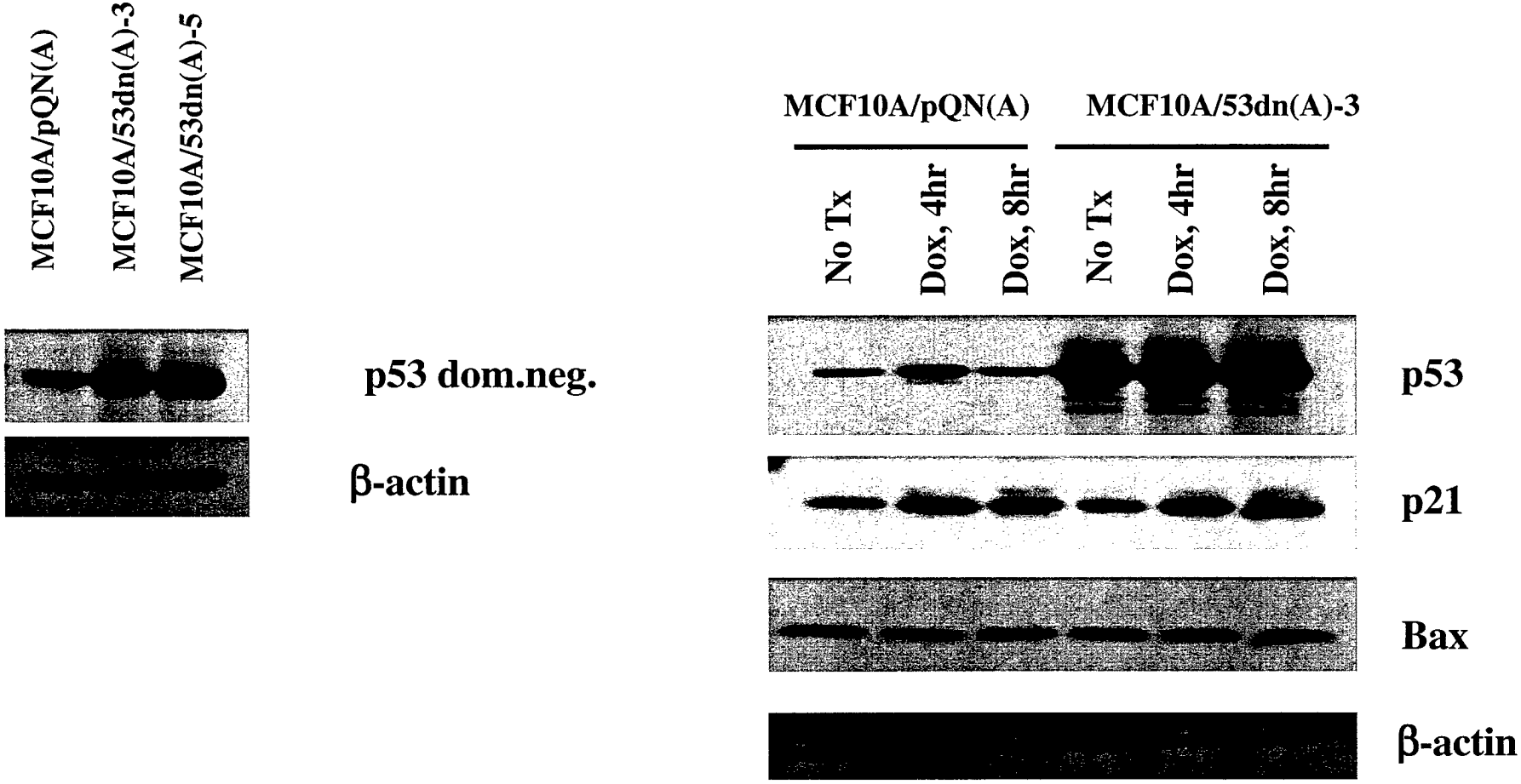


Fig. 7: Generation of MCF-10A cell overexpressing dominant-negative p53. Dominant negative p53 failed to reduce the expression of p53 target genes p21 and Bax

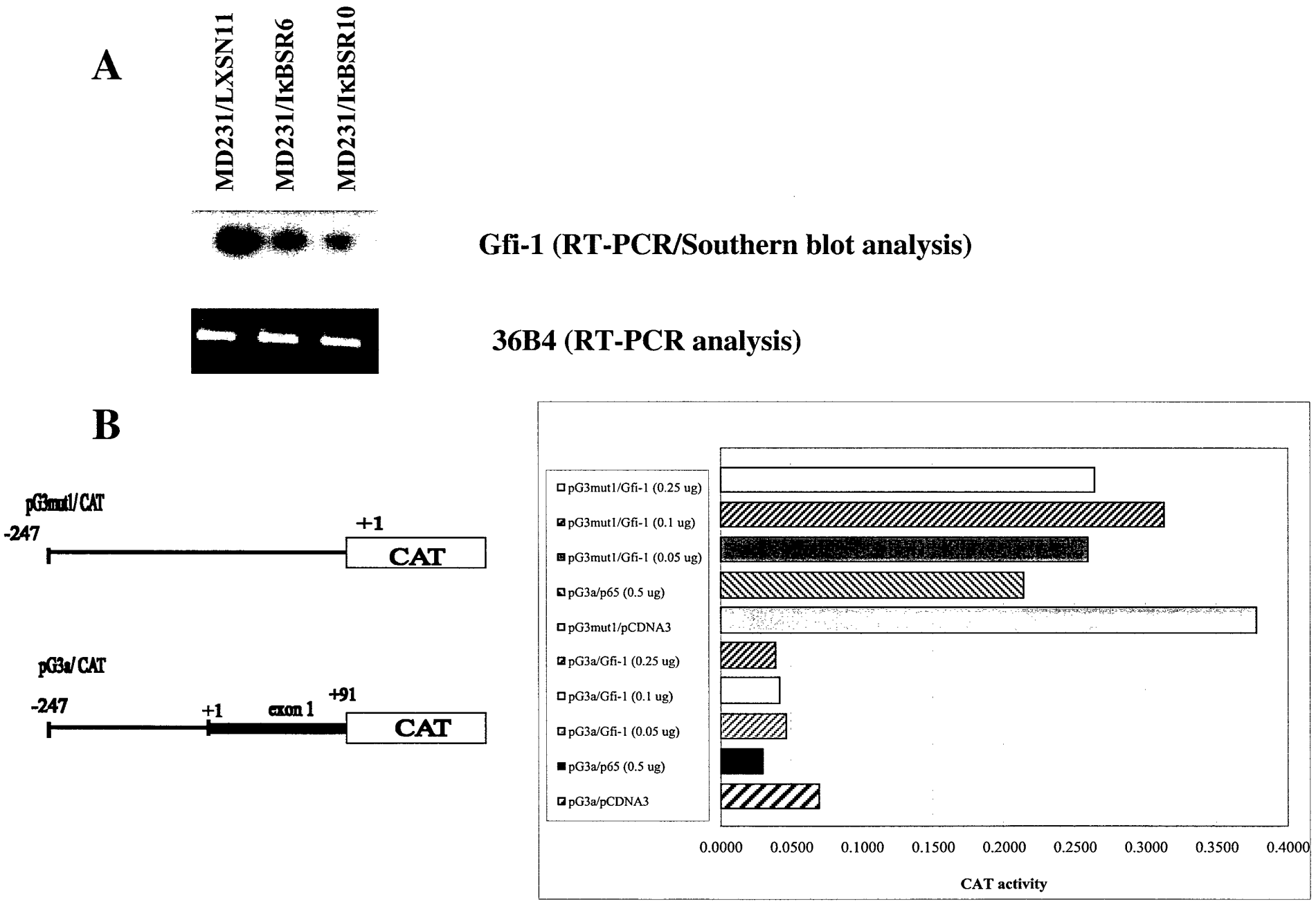


Fig. 8: Gfi-1 is a NF-κB inducible gene. IκBα super-repressor overexpressing cells show lower levels of Gfi-1 (A). Gfi-1 reduces GADD153 promoter activity (B).